Effects of hyperglycemia and aging on nuclear sirtuins and DNA damage of mouse hepatocytes

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Running Title: Hyperglycemia and aging on mouse hepatocytes

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Abbreviations: NOD, non-obese diabetic mice; NAD, nicotinamide adenine dinucleotide;

Abstract

Hyperglycemia, similarly to aging, induces chromatin remodeling in mouse hepatocytes, in comparison to normoglycemia and early-age, respectively. Changes in glucose metabolism also affect the action and expression of sirtuins, promoting changes in chromatin conformation and dynamics. Here we investigated the abundance and activity of the nuclear sirtuins Sirt1, Sirt6 and Sirt7 in mouse hepatocytes, in association with specific histone acetylation, DNA damage and the activation of nucleolar organizing regions (NOR) in hyperglycemic non-obese diabetic (NOD) and old normoglycemic BALB/c mouse strains. Higher levels of Sirt1, PGC-1α and increased expression of gluconeogenesis pathway genes were found in the hyperglycemic NOD mice. Increased Sirt6 abundance was found in the hyperglycemic NOD mice, which might increase DNA damage repair. With aging, lower Sirt1 abundance and activity, increased acetylated histone modifications and Sirt7 levels, and NOR methylation were found. Thus, while

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in normal aging there is reduction in cell metabolism, in the diabetic mice a compensatory mechanism may elevate Sirt1 and Sirt6 levels, increasing gluconeogenesis and DNA repair from the oxidative damage caused by hyperglycemia. Therefore, understanding the regulation of epigenetic factors in diabetes and aging is crucial for the development of new therapeutic approaches that could prevent diseases and ameliorate life quality.

**Key-words:** hyperglycemia, aging, sirtuins, hepatocytes

1. **Introduction**

Type 1 diabetes mellitus (T1DM) is an autoimmune disease caused by lymphocyte infiltration in the endocrine pancreas which leads to the destruction of β-cells and consequently to hyperglycemia (Makino *et al*., 1980). This type of diabetes occurs mainly in childhood and puberty and requires daily insulin replacement therapy in addition to diet and physical activity. According to the International Diabetes Federation (IDF), approximately 480,000 children under 15 years of age and a similar number of 15-25 youngsters have T1DM. In the liver, the absence of insulin signaling promotes a deregulation in glucose and lipid metabolism, such as increased ketogenesis, gluconeogenesis and decreased glycolysis, which result in a severe hyperglycemic state (Berg *et al*., 2002; Nelson and Cox, 2004).

Furthermore, changes in cell metabolism may affect the chromatin organization. Mononucleated hepatocytes of severe non-obese diabetic (NOD) mice, for instance, can attain high levels of polyploidy and undergo chromatin remodeling (Mello *et al*., 2009; Ghiraldini *et al*., 2012), suggesting that hyperglycemia affects on the chromatin organization of hepatocytes in a similar, but not identical, manner as found in aged mice (Mello *et al*., 2009; Ghiraldini *et al*., 2012; Moraes *et al*., 2007). Because increased hyperplasia and apoptosis have been reported in
hepatocytes of diabetic rats (Herrman et al., 1999; Ahmed, 2005), it has been hypothesized that hyperglycemic animals might suffer from early aging (Mello et al., 2009; Blazer et al., 2005).

In aging, a number of modifications in gene expression and epigenetic changes is observed, such as loss of DNA methylation, chromatin remodeling and histone modifications to ensure this process (Cao et al., 2001; Sarg et al., 2002; Mehta et al., 2007; Moraes et al., 2007; Sedivy et al., 2007; Nakamura et al., 2010; Shin et al., 2011). In hepatocytes, particularly, there is an increase in chromatin accessibility to micrococcal nuclease digestion and a global loss of DNA methylation with aging (Mehta et al., 2007; Ghiraldini et al., 2012).

Studies on NAD⁺-dependent deacetylase Sirt1, a mammalian ortholog of Sir2 in yeast, have shown that this protein is involved in aging and caloric restriction, increasing lifespan (Blander and Guarente, 2004; Haigis and Guarente, 2006). Although the sirtuin family comprises seven proteins, only Sirt1, Sirt6 and Sirt7 are nuclear residents (Schwer and Verdin, 2008). Because of the sirtuins dependence on the cofactor NAD⁺, they have been appointed as metabolic sensors, once changes in the [NAD⁺]/[NADH] ratio could modulate their activity and, ultimately, chromatin structure and gene expression (Leibiger and Berggren, 2006; Imai, 2009).

Sirt1 deacetylates specific histone sites and non-histone proteins, such as FoxO1 and PGC-1α. In the liver, the transcription factor PGC-1α is deacetylated, working as a bridge between metabolic changes and gene expression, increasing the expression of gluconeogenic and decreasing the expression of glycolytic genes (Rodgers et al., 2008). Sirt6 is another NAD-dependent deacetylase, involved not only in cell metabolism and metabolic diseases such as diabetes, but also with stress resistance, lifespan, aging and inflammation (Pfluger et al., 2008; Van Gool et al., 2009; Xiao et al., 2010). Despite its preferably ADP-ribosyltransferase activity, this sirtuin can deacetylate H3K9 and H3K56 sites, thus controlling the expression of multiple
glycolytic genes (Zhong et al., 2010). Sirt6 is also known to be involved with the DNA damage repair system, or base excision repair (BER), and is usually highly abundant in the presence of high amounts of reactive oxygen species (ROS) (Mostoslavsky et al., 2006; Lombard, 2008; McCord et al., 2009; Zhong et al., 2010). Sirt6-deficient mice present DNA-damage hypersensitivity and genome instability, with progressive hypoglycemia culminating in an aged-like phenotype and subsequent death (Mostoslavsky et al., 2006).

Sirt7, on the other hand, located in nucleoli, is mostly abundant in the liver (Ford et al., 2006). This protein is associated with transcriptionally active rRNA genes and increases RNA-polymerase I activity (Ford et al., 2006; Murayama et al., 2008; Grob et al., 2009, Tsai et al., 2012). In the liver, changes in the NAD⁺/NADH ratio may regulate Sirt7 to couple changing in energy status with levels of rRNA synthesis and ribosome production (Ford et al., 2006). During aging, the increase in rDNA detachment from the nuclear matrix of mouse hepatocytes could indicate a decreased rDNA transcription, reflecting the cellular metabolism conditions (Moraes et al., 2010).

If chromatin organization is affected by hyperglycemia and aging in mouse hepatocytes, the same may be true for the abundance and activity of nuclear sirtuins, as well as their primary targets. Moreover, given the close relationship of Sirt1, Sirt6 and Sirt7 with cell metabolism, glucose homeostasis and DNA integrity, it is important to address the consequences in their action under a metabolic disorder context. Therefore, the aim of this study was to compare the effects of hyperglycemia and those of aging on nuclear sirtuin abundance and activity, histone modification, DNA fragmentation and nucleolar organization in mouse hepatocytes.

2. Results

2.1. Hyperglycemia and aging affect Sirt1 abundance and activity differently.
Sirt1 abundance and mRNA expression were found to be increased in hepatocytes of severely hyperglycemic mice in comparison to normoglycemic controls, whereas old normoglycemic animals presented a lower Sirt1 abundance than the young-adult controls (Fig. 1A, D; Suppl. Fig. 1A).

Sirt1 activity levels, assessed in order to determine whether Sirt1 abundance has an effect on protein activity, revealed no difference between the hepatocytes of the diabetic mice and the normoglycemic Balb/c and NOD controls. Old mice, however, presented a decrease in Sirt1 activity (Fig. 1B). Because Sirt1 activity did not correspond to the increased abundance of this protein in the diabetic animals, the NAD$^+/NADH$ ratio was calculated to evaluate the availability of this molecule. In the hepatocytes from the diabetic mice, the NAD$^+/NADH$ ratio did not differ from the normoglycemic animals, while in the old normoglycemic animals this ratio was decreased compared to the young-adult controls, similarly to the results found for the Sirt1 activity (Fig. 1C). In all experimental conditions, Sirt1 was identified especially in euchromatic and central regions of the nuclei (Suppl. Fig. 2).

2.2. **Abundance of PGC-1α and Sirt1-histone targets in hepatocytes of diabetic and normoglycemic old mice**

Both diabetic and normoglycemic old mice presented the same PGC-1α abundance pattern in hepatocytes as that found for Sirt1; increased and decreased PGC-1α levels in diabetic and old mice, respectively, were found only in comparison to their controls (Fig. 2A, B). Like Sirt1, PGC-1α was observed in euchromatic and central regions of the hepatocyte nuclei in all experimental conditions (Suppl. Fig. 2). mRNA of target molecules of PGC-1α was also analyzed in order to evaluate if increased PGC-1α abundance reflected on changes in gene
expression. Pck1 and Foxo1 are positively regulated by PGC-1α (Puigserver et al., 2003; Herzog et al., 2005). As expected, their expression in hyperglycemic mice increased in comparison to that in normoglycemic animals. In old animals there was no change in the expression of both genes in comparison with young-adult Balb/c mice (Figure 2C, D).

The abundance of acetylated lysine 26 at histone H1 (H1K26Ac), lysine 16 at histone H4 (H4K16Ac), lysine 14 at histone H3 (H3K14ac) and lysine 9 at histone H3 (H3K9Ac), Sirt1-histone targets, was also evaluated (Fig. 3 A-D, F). Acetylated lysine 56 at histone H3 (H3K56Ac), the Sirt6 target, was also analyzed (Fig. 3 E, F). Except for H3K9Ac, no difference in histone modification abundance was found in the hepatocyte chromatin of the hyperglycemic compared to the normoglycemic mice (Fig. 3 C, F). This epigenetic marker was more abundant in the hepatocytes of the hyperglycemic mice. H3K14Ac was highly abundant in the hyperglycemic mice compared to the Balb/c normoglycemic controls, but did not differ from the NOD normoglycemic controls (Fig. 3 D, F). With the exception of H1K26Ac, old mice presented increased abundance of all histone modifications analyzed in comparison to young-adult controls (Fig. 3 A-F). To determine whether the position of these epigenetic marks in different chromatin areas differed between experimental conditions, immunofluorescence analyses were performed. The histone modifications selected were H3K9Ac and H4K16Ac because of their established position and high availability in hepatocyte nuclei. Both marks were located in euchromatic regions throughout the nuclei in all experimental conditions (Suppl. Fig. 2).

2.3. **Hyperglycemia, but not aging, induces increase in Sirt6 abundance and DNA damage in mouse hepatocytes.**
Sirt6 abundance and expression were found to be increased in the hepatocytes of the hyperglycemic mice but less abundant in the hepatocytes of the normoglycemic old mice in comparison to normoglycemic and young-adult animals, respectively (Fig. 4 A, C). To establish a possible connection between Sirt6 abundance and DNA damage, a comet assay was performed. Higher levels of DNA damage and number of damaged hepatocytes were found in the diabetic mice in comparison with the normoglycemic mice of both strains (Table 2). However, no differences were found in the normoglycemic aged mice compared to young-adults for either of both parameters. The treatment with hydrogen peroxide (H₂O₂) used as positive control for the comet assay, induced increase in DNA damage (over than 300%) and a higher percentage of damaged cells in all cases (over than 200%), except in the hyperglycemic mice, which presented a non-significant increase in both parameters (Table 2).

2.4. Both hyperglycemia and aging induce a decrease in the AgNOR-positive area/ nuclear area ratio, but only aging promotes decrease in Sirt7 abundance and increase in rDNA methylation.

Sirt7 abundance and expression in the hepatocytes of diabetic mice did not differ from that found in the normoglycemic controls, but was decreased in the normoglycemic old mice compared to the young-adults (Fig. 4 B-C). To establish a possible connection between Sirt7 and nucleolar activity, the AgNOR assay was performed to stain specific nucleolar proteins.

Both diabetic adults and normoglycemic old animals presented an increase in nuclear area in comparison with their respective controls, but this increase was more evident in the hepatocytes of the hyperglycemic mice (Fig. 5 A, D). These mice, however, presented no differences in AgNOR-positive areas compared to normoglycemic animals, whereas normoglycemic NOD and Balb/c mice differed with regard to this parameter (Fig. 5 B, D). Since
the hepatocytes of the old mice presented a decrease in AgNOR-positive areas as compared to the young-adult animals, the AgNOR-positive area/nuclear area ratio decreased for both diabetic and old mice compared to normoglycemic and young-adult animals, respectively (Fig. 5 C, D).

In order to understand if the decrease in AgNOR-positive areas in the hepatocytes of old mice was related to DNA methylation, a Native-Chip assay was performed to identify the abundance of DNA methylation in the 18S rDNA region. The diabetic animals did not present any difference in DNA methylation in the 18S rDNA region as compared to the normoglycemic animals (Fig. 6). However, the old normoglycemic BALB/c mice presented a significant increase in this epigenetic mark in comparison to the young-adult normoglycemic mice (Fig. 6).

3. Discussion

Hyperglycemia and aging have been found to promote chromatin remodeling (Moraes et al., 2007; Mello et al., 2009). Although changes in polyploidy and chromatin packing states have been observed in both cases, their magnitude is different (Ghiraldini et al., 2012). The results of the present study indicate that, among other epigenetic proteins involved in the modulation of chromatin functions, sirtuins play a role in coordinating changes in chromatin organization and function in the hepatocytes of diabetic and old mice.

Sirt1, in association with the transcription factor PGC-1α, modulates the gluconeogenesis and glycolysis pathway in the liver, acting as a nutritional sensor (Rodgers et al., 2008). PGC-1α binds either to transcription factors, like FoxO1, which activate genes involved in gluconeogenesis in hepatocytes (Puigserver et al., 2003), or to gene sequences responsible for transcription of key proteins of gluconeogenesis, like the enzyme phosphoenolpyruvate carboxykinase 1 (Pck1) (Herzog et al., 2005). In the present study, Sirt1 and PGC-1α were more abundant in the hyperglycemic mice than in the normoglycemic controls and, as expected, PGC-
α targets were also more expressed in hyperglycemic than in the normoglycemic mice. Although the Sirt1 activity level was not as increased as expected when compared to the protein abundance, the analysis of the NAD\(^+\)/NADH ratio shown here indicated that this molecule could be limiting Sirt1 activity. In the diabetic animals, the limitation of the NAD\(^+\) levels could be due to the higher rate of β-oxidation that occurs in hepatocytes in the absence of insulin signaling (Berg et al., 2002). In untreated cases of T1DM, the absence of insulin signaling could be interpreted by the cell as a fasting state (Nelson and Cox, 2004), despite glucose availability, leading to an increase in sirtuin abundance but not in its activity. In the liver, especially, glucose in excess could reduce this protein activity as well as glycolysis (Chen et al., 2008). In old animals, the decrease in Sirt1 abundance and activity and NAD\(^+\)/NADH ratio levels could indicate a general decrease in cell metabolism (Shin et al., 2011; Gonzalo, 2010). Other authors (Spindler et al., 2003; Braidy et al., 2011) have also reported a decrease in gluconeogenesis and ketogenesis accompanied by a decrease in NAD\(^+\)/NADH with aging, similar to those observed here.

The histone modification H3K9Ac was the only Sirt1-histonic target highly abundant in both diabetic and old mice hepatocytes in comparison with normoglycemic animals from both strains and young-adults, respectively. H3K9Ac, specifically, is a hallmark for gene expression activation (Nakamura et al., 2010). The abundance of this histone modification in diabetic mice could indicate a differentiated gene expression pattern that enables the organism to adapt to long periods of insulin absence. All the histone modifications analyzed, with the exception of H1K26Ac, were more abundant in old animals, indicating that the decrease in Sirt1 abundance and activity might be related to an increase in the remaining acetylated histone sites, which might contribute to a more unraveling chromatin state.
Like Sirt1, Sirt6 is a nuclear protein whose target is also the histone site H3K9Ac, besides the H3K56Ac modification (Michishita et al., 2009; Yang et al., 2009). Interestingly, the Sirt6 abundance in diabetic and old mice was similar to that observed for Sirt1, probably because Sirt1 and Foxo3a act directly on the Sirt6 gene, modulating its expression and consequently glycolysis, triglycerides synthesis and fat metabolism rates (Kim et al., 2010). Sirt6 knockout mice present hypoglycemia by failing to repress the transcription factor Hifα, increasing the glucose capture and glycolysis rate (Zhong et al., 2010). In the present work, Sirt6 was found to be highly increased in the hyperglycemic animals, a finding that could be related to the decreased glycolysis rate in diabetes. In diabetes, the misbalance in the ketogenesis process leads to an accumulation of high amounts of ROS that could generate DNA damage (Berg et al., 2002) and it is known that Sirt6 might play a role in the BER machinery (Mostoslavsky et al., 2006). Indeed, in the hyperglycemic animals a great abundance of Sirt6 and a high percentage of damaged cells and elevated DNA damage index were found, as high as those of the positive control. Sirt6 abundance was lower in the old mice, similar to Sirt1. The reduction in Sirt6 abundance, followed by the increase of its histone targets, H3K9Ac and H3K56Ac, is in accordance with the literature, once that, Sirt6 has been related to early aging in knockout mice (Tennen and Chua, 2011).

Sirt7 is also a nuclear sirtuin occurring mostly in nucleoli and is involved with the activation of RNA polymerase I (Ford et al., 2006). However, there was no report on the relative abundance of Sirt7 and NOR activation in diabetes and aging context. In the diabetic animals, Sirt7 abundance was not different from that in the normoglycemic animals, while in the old mice it was decreased. Active NORs are usually observed in cycling cells and this could be identified by the AgNOR technique due to nucleolar protein retention (McKeown and Schaw, 2009). In old
mouse hepatocytes, rDNA inactivation occurs, mainly because of the detachment of these DNA sequences from the nuclear matrix (Moraes et al., 2010). The decrease in the AgNOR-positive/nuclear area ratio that we observed in both the diabetic and the old animals was promoted by different factors: while in the diabetic mice there was a significant increase in nuclear area, in the old mice there were both an increase in nuclear area and a decrease in AgNOR-positive areas. It has already been established that diabetic and old animals present an increase in nuclear area, mainly because of the increase in ploidy and a decrease in total RNA levels (Moraes et al., 2007; Mello et al., 2009). The decrease in AgNOR-positive areas in old mouse hepatocytes might be related not only to the diminishment in cellular metabolism, but also to the increase in 18S rDNA methylation, observed in this work and in others (Swisshelm et al., 1990; Machwe et al., 2000).

In conclusion, there is a generalized decrease in liver cell metabolism with aging that affects the gluconeogenesis and glycolysis pathways, leading to a decrease in the production of NAD$^+$ and rRNA. We assume that this reduced NAD$^+$ production could induce a decrease in sirtuins abundance. In T1DM, the early-aged phenotype presented by hyperglycemic animals is a response to the absence of insulin signaling from diverse metabolic processes. In the liver, it could activate the Sirt1-PGC-1α pathway increasing the expression of genes related to the gluconeogenesis pathway, besides the DNA repair machinery induced by Sirt6. Therefore, new studies that underlie specifically the epigenetic regulation in diabetes and aging is crucial to a better understanding of complex diseases, such as diabetes mellitus, and discover of new therapeutical approaches that might prevent aging diseases, such as Alzheimer’s, thus ameliorating life quality.

4. Materials and Methods
4.1. Animals

Female NOD/Unib and BALB/cAnUnib mice were obtained from the Multidisciplinary Center of Biological Investigation (CEMIB) of the University of Campinas (Brazil). The animals were reared under standard controlled conditions, fed extruded chow (Nuvital®, Colombo, Brazil) and received water *ad libitum*.

The glycemia levels of the NOD and BALB/c mice were measured once a week, up to 24 h before they were euthanized. Blood samples were obtained by caudal puncture and analyzed using the automatic Accu-Check Active glucose meter (Roche Diagnostica do Brasil, Jaguare, SP, Brazil) that measures glycemia in the range of 10-600 mg/dL or 0.6-33.3 mmol/L. Glycemia levels within the 90-100 mg/dL (5.00-5.55 mmol/L) range were considered normal; glycemia levels over 500 mg/dL (27.5 mmol/L) were considered indicative of severe hyperglycemia. A histological evaluation of the pancreas of the NOD mice was performed, in order to confirm the hyperglycemic status of these animals.

Four mouse groups were used in this study: 1) normoglycemic young (8-week-old) NOD adults, 2) normoglycemic young (8-week-old) BALB/c adults, 3) severe hyperglycemic NOD adults, and 4) normoglycemic 56-week-old BALB/cAnUnib mice. Normoglycemic BALB/c and NOD mice matched for age for both diabetic groups were used as controls. NOD mice are commonly used as a model to study type I diabetes mellitus, because 80% of the females of this strain develop type I diabetes spontaneously. Therefore, the normoglycemic NOD mice used as a control in this study consisted in those 20% of the female individuals that failed to develop the disease.
The protocols involving animal care and use were approved by the Committee for Ethics in Animal Use of the University of Campinas (registration no. 1608-1) and meet the guidelines of the Canadian Council on Animal Care.

Livers for the molecular assays were frozen in liquid nitrogen and stored at -80°C in MNase until use.

4.2. Nuclei isolation

Liver cell nuclei were isolated according to previously described procedures (Blobel and Potter, 1966; Matunis, 2006). Briefly, the livers were homogenized manually in cold TKM buffer (0.05 M Tris-HCl, pH 7.5; 15 mM KCl, 5 mM MgCl₂, 1 mM PMSF, 2 mM DTT, 10 mM sodium butyrate) with 0.25 M sucrose, and a sucrose gradient was produced with a 2.3 M sucrose TKM buffer. The samples were ultracentrifuged (Beckman®, Brea, CA, USA) at 105,000 g and 4°C for 30 min. The nuclei were then resuspended in 0.25 M sucrose TKM buffer supplemented with 2% Triton X-100 and centrifuged at 800 g for 5 min, in order to eliminate endoplasmic reticulum remnants. The nuclei were kept in 0.25 M sucrose TKM buffer supplemented with 30% glycerol at -20°C until use. Nucleus integrity and purity were checked by phase contrast microscopy and by measuring the absorbance ratio at λ 260 nm/280 nm in a UV Diode Array Spectrophotometer (HP®, Palo Alto, CA, USA), respectively.

4.3. Western Blotting

After quantification by the Bradford Protein Assay, equal amounts of proteins were used to prepare the samples. Briefly, nuclei were lysed in 1x SDS sample buffer (60 mM Tris-HCl,
pH 6.75, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and bromphenol blue) and sonicated. Protein was fractionated on a 12% SDS polyacrylamide gel for Sirt1, Sirt6, Sirt7 and PGC-1α, and on a 17% gel for histone modification analysis. All blots were run with a standard molecular weight to confirm and identify the target-protein weight. Proteins were transferred to a nitrocellulose membrane (Millipore®, Billerica, MA, USA) by electroblotting. The membranes were probed with the following antibodies: anti-Sirt1 (H300 - Santa Cruz®, Santa Cruz, CA, USA and Millipore®), anti-Sirt6 (Abcam®, Cambridge, UK and Millipore®), anti-Sirt7 (Abcam® and Millipore®), anti-PGC-1 (Millipore®), anti-acetyl-histone H3 (Lys9) (Millipore®), anti-acetyl-histone H3 (Lys 14) (Millipore®), anti-acetyl-histone H1.4 (Lys 26) (Sigma®, St. Louis, MO, USA), anti-acetyl-histone H3 (Lys 56) (Millipore®) and anti-acetyl-histone H4 (Lys 16) (Abcam®). As loading controls, anti-histone H4 (Millipore®) and anti-histone H3 (Millipore®) antibodies were used. Detection and enhancement were performed by ECL chemiluminescence (Amersham®, Pittsburgh, PA, USA). The membranes were exposed to hyperfilms ECL (Amersham®), and the resulting images were quantified by densitometry with ImageJ® software. The results were normalized with the loading control bands for each gel and expressed as relative abundance to the Balb/c young-adult group.

4.4. Immunofluorescence

Immediately after decapitation, the livers were removed, placed in cold 0.9% NaCl solution and sliced. Imprints on glass slides freshly prepared from the liver slices were fixed in cold 4% paraformaldehyde for 10 min and rinsed three times in cold PBS for 5 min each. The samples were permeabilized with 0.2% Triton X-100 in PBS buffer and blocked with 5% fetal bovine serum in PBS. The following primary antibodies were used: anti-PGC-1 (Millipore®), anti-Sirt1 (Santa Cruz®), anti-acetyl-histone H3 (Lys9) (Abcam®), and anti-acetyl-histone H4
(Lys16) (Abcam®). Sheep anti-rabbit IgG fluorescein conjugated and goat anti-mouse IgG rhodamine conjugated secondary antibodies were purchased from Millipore. Slides were counterstained with DAPI (Sigma®) and mounted in Vectashield medium (Vector Labs®, Burlingame, CA, USA). The images were observed under a Zeiss Axiophot 2 microscope (Carl Zeiss, Oberkochen, Germany) equipped for epifluorescence with an HBO-100W stabilized mercury lamp as light source.

4.5. **Sirt1 deacetylase activity assay**

Equal amounts of total protein from liver nuclei were used for each experimental condition. Positive (resveratrol) control was included for young-adult Balb/c samples using reagents from the SIRT1 Assay Kit (Sigma®, CS1040) according to the manufacturer’s instructions. Sirt1 deacetylase activity was measured using fluorescence intensity signals at 460 nm (excitation 365 nm) was captured in a Fusion microplate fluorometer (Packard®, Meridien, MS, USA). Experimental values were represented as relative activity to Balb/c young-adult mice.

4.6. **NAD⁺/NADH assay**

Twenty milligrams of liver tissue were homogenized, and total NAD⁺ and NADH levels were determined according to the protocol provided in the NAD⁺/NADH Assay Kit (Abcam®). Following the manufacturer’s instructions, total NAD (NAdt) and NADH were detected in a 96-well plate, and color was developed and read at 450 nm in an ELISA plate reader (VersaMax™, Sunnyvale, TX, USA). The NAD/NADH ratio was calculated according to the formula [NAdt – NADH]/NADH.

4.7. **Comet Assay**
The alkaline procedure described by Singh et al., (1988) was used, with modifications required by the material. Fresh livers were minced in Hanks buffer (0.137 mM NaCl, 5.4 mM KCl, 0.25 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 1.3 mM CaCl₂, 1 mM MgSO₄, 4.2 mM NaHCO₃, 1% glucose). To check cell quality, cell viability was determined using the Trypan Blue dye exclusion assay (viability below 70% implies exclusion of the sample) (Collins et al., 1995). For each sample, a positive control with cells treated with hydrogen peroxide (H₂O₂) (100 µM for 30 min at 4°C) was included. All steps described were performed in the dark, to prevent DNA damage by ultraviolet irradiation.

The samples were diluted in 0.5% low-melting-point agarose and pipetted onto slides covered with 1.5% normal-melting-point agarose (Sigma®). The samples were gently spread by placing a coverslip on top, and allowed to solidify at 4°C for 10 min. After removal of the coverslip, the slides were immersed in freshly prepared lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, with 1% Triton X-100 and 10% DMSO) for 24 h at 4°C. After that, the slides were left in the electrophoresis solution (1 mM EDTA and 300 mM NaOH, pH 13, at 4°C) for 30 min, to allow DNA unwinding and expression of alkali-labile damage, followed by electrophoresis (25 V, 300 mA for 20 min at 4°C). The slides were then washed three times in Tris buffer (0.4 M Tris, pH 7.5) and stained with ethidium bromide (20 µg/mL) (Sigma®). Nucleoids were evaluated visually in a blind test [37, 38], using a Zeiss Axiophot 2 microscope equipped for fluorescence microscopy (excitation filter, λ = 450 nm; barrier filter, λ = 490 nm). One hundred comets were classified and assigned to five categories (0-4) according to the extent of DNA migration, as previously described (Jaloszynski et al., 1997; Ghiraldini and Mello, 2010). The extent of DNA damage (D) was determined using the formula D = (L1 + 2xL2 +
\[3xL3 + 4xL4)/(\Sigma/100),\]

where \(L1-L4\) is the number of nucleoids in classes 1-4, respectively, and 
\(\Sigma\) is the sum of all nucleoids counted, including category 0 (no tail).

4.8. **AgNOR staining assay**

AgNOR (silver-stained nucleolar organizer regions) is a cytochemical method based on the specific argyrophilic affinity for some nucleolar proteins, such as B23, nucleolin, UBF and some RNA polymerase I subunits (Rueschoff et al., 1990). Imprints freshly prepared from liver slices on glass slides were fixed in a mixture of absolute ethanol and glacial acetic acid (3:1, v/v) for 1 min, rinsed in 70% ethanol for 5 min and air dried. AgNOR staining was done as described by Rueschoff et al. (1990) and Derenzini and Ploton (1991), preceded by treatment with a 1% Triton X-100 solution in the presence of 4 M glycerol for 15 min at 37°C (Vidal and Mello; 1995; Mello and Vidal, 2008). Briefly, the cells were treated with a solution containing 2 volumes of 50% aqueous silver nitrate (Merck®, Readington, PA, USA) and 1 volume of 2% gelatin in 1% aqueous formic acid (v/v). The aqueous solutions were prepared with deionized water. The optimal staining time was found to be 10 min at 37°C.

4.9. **Native Chromatin immunoprecipitation**

Micrococcal nuclease digestion (30U/mg DNA) of native chromatin from isolated hepatocyte nuclei was performed for 10 min at 25°C and stopped with EDTA (15 mM). After brief centrifugation, supernatant 1 (SN1) was collected and the digested chromatin was resuspended, released in TEEP buffer (10 mM Tris pH 7.5; 0.5 mM EDTA; 0.5 mM EGTA; 250 
\(\mu\)M PMSF, and 0.05% Triton-X) with 5 mM NaCl, and left overnight at 4°C. Samples were centrifuged for 30 s at 5000 rpm and supernatant 2 (SN2) was collected. Each ChIP reaction and input was performed with 5 \(\mu\)g of chromatin (2.5 \(\mu\)g from SN1 and 2.5 \(\mu\)g from SN2) using specific 5 methyl-cytidine (Abcam®) and IgG anti-mouse (Santa Cruz®) monoclonal antibodies.
for mock control. The hybridization of the complex antibody and magnetic beads (Invitrogen®, Carlsbad, CA, USA) with the chromatin was done overnight, with constant rotation at 4°C. The samples were washed twice in TEEP buffer with 140 mM and 200 mM of NaCl, respectively, and eluted in TEEP buffer with 50 mM NaCl and 1% SDS.

4.10. Nucleic acid extraction and qPCR

DNA was extracted from the immunoprecipitated samples using phenol/chloroform. Primers were designed to amplify a 102 pb product from the 45S gene that corresponds to the 18S rDNA region. To analyze gene expression and corroborate the Western Blotting results for nuclear sirtuins, RNA was extracted with the RNeasy Mini Kit (Qiagen®, Hilden, Germany) from mouse livers stored in RNLater® (Ambion, Austin, USA). All the primers used to quantify the expression of nuclear Sirts, targets of PGC-1α and 18S gene, are described in Table 1. For the qPCR assay, the FastStart Universal Syber Green Master (ROX) mix (Roche®, Penzberg, Germany) was used in an ABI 7500 thermocycler (Applied Biosystems®, Foster City, CA, USA).

4.11. Statistics

All statistical analyses were performed with the Minitab 12™ software (State College, PA, USA). Data comparison between the diabetic animals and their controls, resulting from five independent sets of experiments, was performed by means of the ANOVA test, while for pairwise comparison Student’s t-test was used. P<0.05 was considered the critical level for rejection of the null hypothesis.

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6. References


Kim, H et al. (2010). C. Deng, Hepatic-specific disruption of Sirt6 in mice results in fatty liver formation due to enhanced glycolysis and triglyceride synthesis. Cell Metab. 12, 224-236.


Table 1 – Primers used for gene expression and Native-ChIP assay

<table>
<thead>
<tr>
<th>Assay</th>
<th>Gene</th>
<th>Primer Forward</th>
<th>Primer Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene expression</td>
<td>Sirt1</td>
<td>CTCCTGTGTTGACCGATGGACT</td>
<td>ATCGGTGCAATCATGAGAT</td>
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<tr>
<td></td>
<td>Sirt6</td>
<td>CCTGTAGAGGGGAGCTGAGA</td>
<td>GAGGTACCCAGGGTGACAGA</td>
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<tr>
<td></td>
<td>Sirt7</td>
<td>GAGAGCGAGGATCTGGTGAC</td>
<td>GCCCGTGTAAGACAACCAAGT</td>
</tr>
<tr>
<td></td>
<td>Foxo1</td>
<td>GCTGGGTTGTCAGGCTAAAGAG</td>
<td>TGGCCAAGTTCTGAGGAAAGG</td>
</tr>
<tr>
<td></td>
<td>Pck1</td>
<td>CCTGGAAGAACAAAGGAGTGG</td>
<td>CTACGGCCACCCAAAGATGAT</td>
</tr>
<tr>
<td>Native-ChIP</td>
<td>18S</td>
<td>CGAAAGCATTTGCCAAAGAT</td>
<td>AGTCGGCATCTGTATGGATC</td>
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</table>
Table 2 – Percentage of damaged cells and DNA Damage Index in mouse hepatocytes after H$_2$O$_2$ treatment

<table>
<thead>
<tr>
<th>Animals</th>
<th>H$_2$O$_2$</th>
<th>Damaged cells (%)</th>
<th>DNA Damage Index</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>X</td>
<td>SE</td>
<td>X</td>
<td>SE</td>
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<tr>
<td>Young-Adult Balb/c</td>
<td>No</td>
<td>21.38</td>
<td>4.7</td>
<td>34.72$^a$</td>
<td>8.60</td>
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<td></td>
<td>Yes</td>
<td>53.75*</td>
<td>14.6</td>
<td>111.97*</td>
<td>29.50</td>
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<tr>
<td>Normoglycemic Balb/c</td>
<td>No</td>
<td>20.67$^a$</td>
<td>3.0</td>
<td>37.58$^a$</td>
<td>6.11</td>
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<td>Yes</td>
<td>53.40*</td>
<td>12.4</td>
<td>105.09*</td>
<td>24.30</td>
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<tr>
<td>Normoglycemic NOD</td>
<td>No</td>
<td>22.58$^a$</td>
<td>4.1</td>
<td>40.89$^a$</td>
<td>9.32</td>
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<td>Yes</td>
<td>56.30*</td>
<td>11.5</td>
<td>128.72*</td>
<td>31.50</td>
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<tr>
<td>Hyperglycemic NOD</td>
<td>No</td>
<td>43.78$^b$</td>
<td>3.8</td>
<td>84.23$^b$</td>
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<td>61.57</td>
<td>13.8</td>
<td>148.77</td>
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<td>Old Balb/c</td>
<td>No</td>
<td>25.46$^a$</td>
<td>2.6</td>
<td>48.34$^a$</td>
<td>6.27</td>
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<td>63.09*</td>
<td>14.5</td>
<td>159.75*</td>
<td>30.60</td>
</tr>
</tbody>
</table>

X, arithmetic means; SE, standard error; *, difference from H$_2$O$_2$ treatment significant at P<0.05 (Student’s t Test); a,b, different letters in the same column indicate statistical differences significant at P<0.05 (ANOVA).
**Figure 1.** Sirt1 is differently affected in hepatocytes of diabetic adults and normoglycemic old mice. (A) Sirt1 relative abundance in young-adult BALB/c mouse hepatocyte nuclei as determined by Western Blotting. (B) Sirt1 relative activity in hepatocyte nuclei compared to young-adult BALB/c, determined by the Sirt1 Activity Assay Kit. Positive control, resveratrol (12.5 mM). (C) NAD⁺/NADH ratio in mouse hepatocytes. (D) Sirt1 representative image of the immunoblotting. Image was chosen to specifically highlight the differences shown in the graph. Histone H3 was used as loading control. B, BALB/c; N, NOD; + hyperglycemia; - normoglycemia. *, difference significant at P<0.05 by ANOVA. †, difference significant at P<0.05 by Student’s t test.
Figure 2. PGC-1α presents the same abundance pattern as Sirt1 and activates its targets in hyperglycemic animals. (A) PGC-1α relative abundance, determined by Western Blotting. *, difference significant at P<0.05 by ANOVA; †, difference significant at P<0.05 by Student’s t test. (C) PGC-1α representative image of the immunoblotting. Image was chosen to specifically highlight the differences shown in the graph. B, BALB/c; N, NOD. Histone H3 was used as the loading control. +, hyperglycemia; -, normoglycemia; (C) Pck1 (D) Foxo1 expression, determined by qPCR. *, difference significant in comparison to normoglycemic NOD mice at P<0.05 by Student’s t test.
Figure 3. Abundance of Sirt1 histone targets in diabetic and old mouse hepatocytes. (A) H1K26Ac, (B) H4K16Ac, (C) H3K9Ac, (D) H3K14Ac, (E) H3K56Ac. The abundance of Sirt1 and Sirt6 histone targets was determined in WB using young-adult BALB/c hepatocyte nuclei as control. (F) Representative image of the immunoblotting of H1K26Ac, H4K16Ac, H3K9Ac, H3K14Ac, H3K56Ac. Image was chosen to specifically highlight the differences shown in the graph. B, BALB/c; N, NOD. Histone H3 was used as the loading control for histone H4 and H1 modifications, and H4 was used as the loading control for histone H3 modifications. +,
hyperglycemia; -, normoglycemia; *, difference significant at P<0.05 by ANOVA; †, difference significant at P<0.05 by Student’s t test.
**Figure 4.** Abundance of Sirt6 and Sirt7 in diabetic and old mouse hepatocytes. (A) Sirt6, (B) Sirt7. Sirtuin abundance was calculated in WB using young-adult BALB/c hepatocyte nuclei as control. (C) Sirt6 and Sirt7 representative image of the immunoblotting. Image was chosen to specifically highlight the differences shown in the graph. Histone H4 was used as loading control. B, Balb/c; N, NOD; +, hyperglycemia; -, normoglycemia. *, difference significant at P<0.05 by ANOVA; †, difference significant at P<0.05 by Student’s t test.
Figure 5. Nuclear and AgNOR-positive areas in diabetic and old mouse hepatocytes. (A) Nuclear area; (B) AgNOR-positive area in imprinted mouse hepatocytes. (C) AgNOR area relative to nuclear area. +, hyperglycemia; -, normoglycemia; *, difference significant at P<0.05 by ANOVA; †, difference significant at P<0.05 by Student’s t test. (D) AgNOR-stained hepatocyte: 1- Young-Adult Balb/c; 2- Old Balb/c; 3- Hyperglycemic NOD; Bars, 10µm.
Figure 6. Native ChIP from mouse hepatocytes as % of input. IgG was used as mock control. †, difference significant at P<0.05 by Student’s t test.